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Review

Homologous recombinational repair of DNA ensures mammalian chromosome stability

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Abstract

The process of homologous recombinational repair (HRR) is a major DNA repair pathway that acts on double-strand breaks and interstrand crosslinks, and probably to a lesser extent on other kinds of DNA damage. HRR provides a mechanism for the error-free removal of damage present in DNA that has replicated (S and G2 phases). Thus, HRR acts in a critical way, in coordination with the S and G2 checkpoint machinery, to eliminate chromosomal breaks before the cell division occurs. Many of the human HRR genes, including five Rad51 paralogs, have been identified, and knockout mutants for most of these genes are available in chicken DT40 cells. In the mouse, most of the knockout mutations cause embryonic lethality. The *Brca1* and *Brca2* breast cancer susceptibility genes appear to be intimately involved in HRR, but the mechanistic basis is unknown. Biochemical studies with purified proteins and cell extracts, combined with cytological studies of nuclear foci, have begun to establish an outline of the steps in mammalian HRR. This pathway is subject to complex regulatory controls from the checkpoint machinery and other processes, and there is increasing evidence that loss of HRR gene function can contribute to tumor development. This review article is meant to be an update of our previous review [Biochimie 81 (1999) 87]. Published by Elsevier Science B.V.

Keywords: Homologous recombinational repair; DNA; Chromosome

1. Introduction

This review is a sequel to the article we wrote less than 2 years ago [1] in an area of DNA repair and cancer biology that is developing very rapidly. Here we emphasize current findings for the proteins that participate directly in homologous recombinational repair (HRR), its regulation within the framework of cell cycle checkpoints, and its involvement in human cancer. We cite previously referenced publications only in some instances to reiterate certain points. Other recent reviews emphasize various aspects of homologous recombination in the context of double-strand break (DSB) repair in mammalian cells [2–13] or yeast [14,15].

Genomic instability can be viewed from various perspectives, but here we are particularly concerned with the recombinational repair processes, which

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are responsible for DNA-strand integrity at the level of the whole chromosome. A distinguishing feature between normal diploid cells and most cancer cells is the ability to avoid chromosomal rearrangements, i.e. translocations, deletions, duplications, and inversions. These abnormalities typically associated with cancer cells arise when the DSB repair systems have failed, either directly, or as a consequence of defects in the coupling of repair with modulation in cell cycle progression. DSBs in vertebrates are repaired by both non-homologous end joining (NHEJ) and HRR [5,12,16–18]. Until recently, NHEJ was thought to be the primary mechanism in mammalian cells for repairing DSBs, which are the principal lethal lesion produced by ionizing radiation (IR). This view was supported by the fact that rodent-cell mutants for several genes (*Ku70*, *Ku86*, *DNA-PKcs*, *LIG4*, and *XRCC4*) participating in NHEJ show high sensitivity (up to ~6-fold) to cell killing and induction of chromosomal aberrations in response to IR [19]. These mutants show defective rejoining of IR-induced DSBs although their deficiencies are incomplete [19].

Only recently were mutants derived from screening procedures identified [20,21] as being defective in the HRR pathway, which is mediated by the highly conserved Rad51 strand transferase (see Fig. 1 for participating proteins). In particular, the *xrcc2* and *xrcc3* hamster lines have significant (~2-fold) IR sensitivity, and they show no detectable defects in IR-induced DSB repair in electrophoretic assays employing supra-lethal doses (>10 Gy). Nevertheless, as indicated by chromosomal aberrations, the *xrcc2* and *xrcc3* mutants experience high levels of spontaneous and IR-induced DSBs [1,22]. Recent studies employing enzymatically produced site-specific DSBs in introduced sequences revealed gross defects in both these mutants for the removal of these DSBs by HRR (discussed in Section 2.1) [23,24].

There is now much evidence that DNA replication generates DSBs that are efficiently repaired through HRR between sister chromatids [15,25]. A single-strand break located immediately in front of a replication fork could be converted to a DSB. Thus, the high level of spontaneous chromosomal aberrations in the *xrcc2* and *xrcc3* mutants may be attributed to a partial deficiency in HRR normally occurring between chromatids that have replicated. A complete deficiency, for example through loss of Rad51 function, leads to loss of cell viability [25–27]. The evidence for a role of NHEJ in eliminating spontaneous DSBs has been paradoxical. CHO *ku86*/*xrcc5* and chicken DT40 *ku70* mutants show little or no increase in chromosomal aberrations [16,28]. In contrast, *ku86* knockout mice show vastly elevated levels of chromosomal aberrations in cultured embryonic and adult-skin fibroblasts (56 and 83% of metaphase cells, respectively) [29,30]. Curiously, the extremely

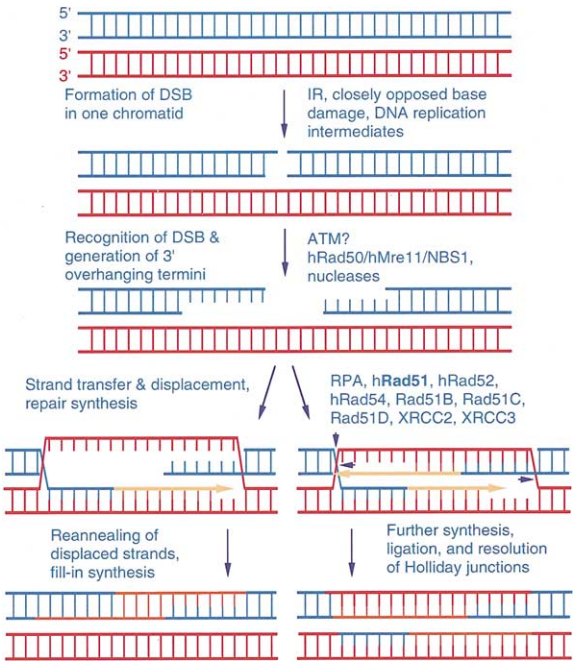

Fig. 1. Sequence of events during homologous recombinational repair of a DSB. The proteins that initially recognize DSBs and recruit the recombination proteins have not been defined, nor have the particular polymerases, ligases, and nucleases. The right-hand pathway at the bottom depicts classical reciprocal exchange involving the resolution of Holliday junctions to produce repair patches in both chromatids (shown by horizontal arrows). Resolving a Holliday junction in the opposite orientation (vertical arrowhead) to that shown will yield a crossover product, which will result in a cytologically-visible sister-chromatid exchange. However, reciprocal-exchange products were not observed during gene conversion between sister chromatids [39], suggesting that the major pathway of DSB repair in mammalian cells involves synthesis-dependent strand annealing [15,171] as shown on the left. In this case, all newly synthesized DNA resides in the initially damaged chromatid.

poor growth of *ku86*$^{-/-}$ MEFs [30] appears inconsistent with the fact that these cells were derived from embryos that were able to complete development. The *xrcc4*$^{-/-}$ MEFs (mouse embryo fibroblasts) also have very high levels of chromosomal aberrations (~50% of cells) [31].

Both NHEJ [30,32] and HRR (see Sections 2.2 and 6) likely contribute to the suppression of tumorigenesis. During the development of a malignant phenotype, pre-cancerous cells that are compromised for HRR would have to rely more on NHEJ in S and G2 phases to remove DSBs. Because NHEJ is inherently error-prone, this shift would likely generate more chromosomal rearrangements such as translocations [33,34], as well as small deletions and insertions [17]. One can speculate that improperly regulated HRR may promote the loss of heterozygosity (LOH, and unmasking of oncogenic mutations) that is often seen in tumor cells [35]. The repair of DSBs through interaction of homologous chromosomes is much less efficient than between chromatids, which may protect cells from the effects of LOH [12].

Sister-chromatid exchanges (SCEs), which are measured cytologically by differential staining of sister chromatids, are induced by many DNA-damaging agents and have long been speculated to arise from homologous recombination although other models based on NHEJ have also been proposed [36]. Recent studies support a mechanism of homologous recombination in these exchange events. Specifically, spontaneous and mitomycin C-induced SCE levels were significantly reduced in chicken or mouse mutants having diminished levels of Rad51 or Rad54 proteins but were normal in *ku70* mutant cells [37,38]. Thus, a substantial fraction of SCEs likely arise from reciprocal exchange occurring through the resolution of Holliday junctions (see Fig. 1 legend). However, the emerging picture suggests that very few DSBs present in replicated DNA get repaired through a mechanism that results in SCE. Studies utilizing I-*Sce*I endonuclease to create DSBs in direct-repeat substrates indicate that sister-chromatid gene conversion is the predominant pathway of repair and that reciprocal exchange rarely occurs (<3% of repair events) [39]. These findings help explain why IR [40] and restriction enzymes [41,42] are weak inducers of SCEs.

2. Genetic analysis

2.1. Roles of Rad51 paralogs and Rad54 in the repair of DSBs by HRR

The core reactions of homologous pairing, strand-transfer, and strand exchange or strand annealing (see Figs. 1 and 2, right box) likely involve the trimeric single-strand binding protein, RPA, the human homologs of *Saccharomyces cerevisiae* Rad51, Rad52, Rad54, and five proteins that we have designated Rad51 "paralogs". Paralogs are genes that arose through duplication of an ancestral gene and acquired new functions. In this case the paralogs show a high degree of evolutionary divergence from Rad51 as well as from each other. Nevertheless, they are well conserved between mammalian and chicken cells [43,44]. These paralogs are XRCC2 and XRCC3, which were identified by functional complementation of hamster-cell mutants, and the Rad51B/Rad51L1, Rad51C/Rad51L2, and Rad51D/Rad51L3 proteins, whose sequences were identified through database analysis as previously summarized [1].

Recently, direct evidence was obtained showing that XRCC2 and XRCC3 strongly contribute to the repair of DSBs. Recombination substrates were introduced by stable transfection of mutant and wild-type hamster-cell lines. By subsequent transfection of the I-*Sce*I endonuclease, DSBs were produced at a specific site within the target gene (either *neo* or *GFP*, green fluorescent protein), which was present as a direct-repeat of two mutant heteroalleles. Recombination frequencies were reduced ~100- and 25-fold in the *xrcc2* [24] and *xrcc3* mutants, respectively [23]. The specific recombination substrates used in the *xrcc2* study were designed to distinguish short-tract gene conversion (STGC) from long-tract gene conversion plus unequal SCE (these latter two classes were indistinguishable) [24]. In both normal and *xrcc2* mutant cells, the two distinguishable classes of events were approximately equal. The *GFP* substrate in the *xrcc3* study was specifically designed to detect STGC [23]. A subsequent study using an analogous *neo* substrate found that absence of *xrcc3* decreased the HHR frequency ~100-fold [45]. It remains to be determined how accurately these recombination substrates model the repair of DSBs arising in native chromosomal DNA.

Recently, the construction of knockout mutants of *Rad51b*, *Rad51c*, and *Rad51d* in chicken DT40 cells confirmed that each of these proteins has non-overlapping functions. Remarkably, these mutants as well as the *xrcc2* and *xrcc3* DT40 mutants all exhibit very similar phenotypes [43,44]: elevated spontaneous chromosomal aberrations, high sensitivity to killing by cross-linking agents (mitomycin C and cisplatin), mild sensitivity to γ-rays, and defective Rad51 focus formation after exposure to γ-rays. These findings suggest that the Rad51 paralogs participate in repair as a functional unit, which may promote the assembly of Rad51 nucleoprotein filaments (discussed in Section 3.1). Moreover, *xrcc2* and *xrcc3* hamster mutants have chromosome mis-segregation associated with fragmentation of the centrosome [46]. Discussion of mouse knockout mutations in *Rad51* paralogs is given below in the context of other mouse HRR mutations.

Rad54 knockout mutants of mouse embryonic stem (ES) cells have ∼2-fold increased IR sensitivity [47]. In DSB-induction studies analogous to those described above for *xrcc2* and *xrcc3* cells, *Rad54* ES cells showed slightly reduced DSB repair by gene conversion, as well as reduced mitomycin C-induced SCE [38]. These reductions were on the order of 1.5-fold, i.e. much less than seen for the *xrcc2* and *xrcc3* mutants.

2.2. Properties of knockout mutations of HRR genes in the mouse

Table 1 lists the knockout mutations in the HRR pathway that were recently tabulated [48] along with other mutations in related DNA repair responses. Studies with *Rad54*-deficient mice reveal complexities in the pathways of DSB repair [47]. Whereas *Rad54* ES cells and early embryos show IR sensitivity, in adult mice this mutation confers sensitivity only in a *scid/DNA-PKcs* background and only in selected tissues, most notably the bone marrow. These results suggest that the pathways of NHEJ and HRR can have overlapping and specialized roles that change during development.

Table 1
Phenotypes of mouse knockout mutants in the HRR pathway[a]

Gene	Protein function	Phenotype	Ionizing radiation sensitivity
Rad51	Strand transferase	Arrest at ≤ E5	Increased[b]
Rad52	Enhancement of *Rad51* activity; binding DS ends	Viable	Normal
Rad54	ATPase; DNA unwinding; formation or stabilization of heteroduplex	Viable	Increased in ES & embryo cells
Xrcc2	Promotes Rad51 focus formation and chromosome segregation	Arrest at ≥ 12.5[c]	ND[d]
Rad51b	Promotes Rad51 focus formation	Arrest at E6	ND
Rad51d	Promotes Rad51 focus formation	Arrest at E8.5–11.5	ND
Rad50	Enhancement of Mre11 nuclease activity	Arrest at E6	Increased[b]
Nbs1	DNA damage sensor? Complex formation with Mre11/Rad50	Early arrest[e]	ND
$Brca1^{ex11}$	Rad50 focus formation	Arrest at E4.5–9.5	Increased[b,f]
Brca2	Rad51 focus formation	Arrest at E7.5–8.5	Increased[b,g]
$(Brca2^{ex11})$[h]	Rad51 focus formation	Reduced viability	Increased
Atm	Ser/Thr checkpoint kinase	Viable but growth retarded	Increased
Atr[i]	Ser/Thr checkpoint kinase	Arrest at <E7.5	ND

[a] Some of the information on mutant phenotypes was taken from the updated tabular review by Friedberg and Meira [48].
[b] Determined for blastocyst [27,49,54,202].
[c] Thacker and Deans, [223].
[d] ND = not determined.
[e] Chen, personal communication.
[f] Also determined using ES cells and adult fibroblasts [53,66].
[g] Also determined using MEF cultures [55,56].
[h] Disruptions of exon 11 within the BRC repeats sometimes result in viable but runted offspring. MEF cultures carrying these mutations grow poorly and undergo premature senescence [55,56,60].
[i] The *Atr* knockout was very recently reported [192].

Knockout mutations in the *Rad51* gene cause early embryonic lethality with arrest occurring at E5 (embryonic day 5) or even much earlier [26,27]. The null *rad50* mutation is lethal in both cultured ES cells and in early developing embryos at E6 [49]. This result indicates that, like Rad51, the mammalian Mre11/Rad50 protein complex (whose yeast homologs belong to the Rad52 epistasis group of IR sensitivity) has functions in proliferating cells that are essential for viability. Early embryonic *rad50* mutant cells in culture are hypersensitive to IR, consistent with a role for this complex in the repair of IR-induced DSBs [49].

Mouse knockouts have been made in three of the five Rad51 paralogs, and they each exhibit embryonic lethality. Rad51B-deficient embryos arrest at ∼E6, and mutant blastocysts (E3.5) in culture fail to proliferate [50]. Rad51D-deficient embryos arrest significantly later (E8.5–E11.5) and show posterior truncation [51]. Rad51D mutant embryonic cells do not proliferate in culture [51]. Xrcc2-deficient embryos arrest at E12.5 [223]and have high chromosomal abnormalities.

There is increasing genetic evidence that the *Brca1* and *Brca2* genes, which are implicated in hereditary breast and ovarian cancer, regulate or participate in the HRR pathway (see Fig. 2). Complete knockout mutations of both of these genes in mice are lethal in early embryogenesis [52]. Truncating disruption mutations in *Brca1* or *Brca2* in mice are viable although survivors are few and show severe growth deficiency (Table 1). Several studies using *Brca1*-deficient [53,54] and *Brca2*-deficient [55,56] mouse cells show moderately increased sensitivity to IR, as seen in human tumor lines [57–59]. Moreover, both numerical and structural chromosomal aberrations were seen in *Brca1* and *Brca2* mutant embryos [54,60,61] or MEFs [62]. In human MCF7 cells, conditional overexpression of a BRCA2 BRC domain (which differs from the BRCT domain of BRCA1) produced increased sensitivity to IR through a dominant negative effect [63].

Recently, direct genetic evidence supporting a role for Brca1 in HRR was obtained. *Brca1* mouse ES cells carrying an exon 11 truncation ($\Delta^{223-763}$) have ∼5-fold reduced efficiency of homologous repair of I-*Sce*I-induced DSBs, but no defect in NHEJ [64]. Moreover, the gene targeting efficiency was greatly reduced (>10-fold) in these cells, and there was also ∼4-fold increased efficiency of random chromosomal integration of transfected plasmid DNA. Similar results for gene targeting and random integration were found by another laboratory using the same cells [65]. However, attempts to fully complement these mutant cells with a *Brca1* minigene were unsuccessful, reversing only the change in efficiency of random integration [65]. While these findings may have major implications for the role of Brca1 in DSB repair pathways, they should be viewed with the caveat that the single *Brca1* cell line obtained could have acquired secondary changes influencing recombination [66].

3. Biochemistry of HRR

3.1. HsRad51 protein

The eukaryotic Rad51 protein has been shown to be the structural and functional homolog of the prokaryotic RecA strand-transfer protein (reviewed in [1,67–69]). Both RecA and Rad51 proteins contain Walker motifs and bind and hydrolyze ATP while forming a filament on single-stranded DNA and performing strand transfer. ATP (actually ATP-Mg^{2+}) frequently plays a dual role: its binding acts as an allosteric effector, and its hydrolysis serves as an energy source. A *rad51* mutation in the Walker motif A from GK(T/S) to GR(T/S) results in a protein able to bind ATP, but not able to hydrolyze it, while a similar mutation to GA(T/S) results in the inability to both bind and hydrolyze ATP. Studies with such mutations have shown that RecA requires only binding of ATP for strand-exchange activity, but requires ATP hydrolysis in order to complete recombination. Studies using a non-hydrolyzable ATP analog (ATP-γS) in combination with the wild-type RecA protein have shown similar results [68].

Unlike RecA, human Rad51 (HsRad51) mutant protein that can bind but not hydrolyze ATP promotes some completion of recombination both in vitro and in vivo [70]. The in vivo system utilized a DT40 chicken cell line containing a chicken *Rad51* gene knockout, complemented by the *HsRad51* cDNA under the control of the tetracycline-dependent repressor. Under conditions where the wild-type HsRad51 protein was not present, expression of an ATP binding, but non-hydrolyzing mutant HsRad51 complemented these DT40 cells for survival after IR

(implying recombinational repair). However, the defective gene targeting was not complemented [70]. Expression of a HsRad51 protein that cannot even bind to ATP did not complement any aspect of the *rad51* phenotype. Simultaneous in vitro studies show that HsRad51 protein with a mutation preventing ATP hydrolysis binds to DNA less efficiently, but does catalyze strand exchange, consistent with earlier reports that replacing ATP with ATP-γS does not completely block the strand-transfer activity of HsRad51 (reviewed in [70]).

A more detailed in vitro examination of the effects of ATP binding and hydrolysis on HsRad51 activity has been performed, and this work helps explain the difference between HsRad51 and RecA [71]. While neither ATP nor ATP-γS affects the binding of RecA monomers to ssDNA, both NTPs greatly increase the cooperative filament assembly of RecA on ssDNA. On the other hand, HsRad51 forms filaments on ssDNA approximately equally well in the presence or absence of these two NTPs. HsRad51 monomers bound to either NTPs have slightly lower individual affinity for ssDNA, but this is counterbalanced by a slight increase in the cooperative binding to ssDNA. As discussed by De Zutter and Knight [71], two groups [72,73] previously showed that the Rad51 protein from the yeast *S. cerevisiae* (ScRad51) does not bind well to ssDNA in the absence of ATP, indicating a possible major difference between the yeast and human proteins. Ideally, experiments by a single group with both proteins should be done in order to confirm this difference.

Additional in vitro experiments have shown that HsRad51 by itself (i.e. without associated proteins like HsRad52) is less efficient than RecA at promoting DNA strand exchange, and that both proteins are less efficient as the GC content of the DNA increases [74]. At 40% GC, very similar to that of human DNA, HsRad51 is able to form homologous joint molecules between ssDNA and dsDNA, but was unable to complete strand exchange. The authors suggest that HsRad51, in the absence of additional proteins, may act primarily as a pairing protein, as opposed to a strand-transfer protein that can form extensive heteroduplex DNA. A second report also found that the purified HsRad51 protein could not form extensive heteroduplex DNA [75]. Both RecA and ScRad51 can form significantly longer heteroduplex DNA than HsRad51. These authors suggest that HsRad51 may play a role primarily in the initial search for homology and the formation of joint molecules. It is known though that in vivo the HsRad51 protein interacts with a number of additional proteins that appear to assist strand-transfer activity, most notably Rad52, Rad54, and the Rad51 paralogs (reviewed in [1] and below). It was previously shown by several groups that the ssDNA binding protein RPA binds to both human and yeast Rad52, and recently HsRad51 was also found to interact with RPA [76].

HsRad51 also appears to differ from RecA in the regions of the protein involved in DNA binding. In RecA, one of the DNA binding domains lies in the C-terminus (reviewed in [68]), a region not conserved with Rad51. The tertiary structure of the N-terminal domain of the HsRad51 has recently been determined by NMR spectroscopy [77]. This structural analysis strongly suggested that this domain is involved in the binding of DNA, and this interpretation was confirmed by showing that specific mutations in this domain blocked the in vitro binding of HsRad51 to DNA. The authors point out that their results are consistent with evidence that c-Abl kinase can inhibit HsRad51 binding to ssDNA by phosphorylating Tyr^{54} in the N-terminal domain [78]. While the N-terminal part of HsRad51 appears to be involved in DNA binding, this region does not appear to be involved in the interaction with HsRad52, which is reported to require the C-terminal third of HsRad51 [79]. Earlier evidence from two-hybrid studies on the interaction of the yeast proteins indicated that the interacting domain was in the N-terminal region of ScRad51 [80]. Further experiments will be needed to determine if this is a real difference between HsRad51 and ScRad51.

There have been conflicting reports in the last few years on the in vitro polarity (i.e. the end of the ssDNA that is transferred into the dsDNA) of HsRad51- and ScRad51-dependent strand-transfer. Several studies have suggested that the polarity of the eukaryotic proteins is different from that of RecA (reviewed by [81]). A recent study suggests that the yeast Rad51 protein can promote strand-transfer with either polarity if the reaction uses a dsDNA substrate having either a 5′ single-strand tail, or a 3′ single-strand tail (as appears to be the case in vivo) instead of ssDNA, which is often used in model systems [81].

3.2. HsRad52 protein

The purified human Rad52 protein is known to assist HsRad51 in forming joint molecules between ssDNA and homologous dsDNA and to promote annealing of complementary strands in the absence of HsRad51 (reviewed in [1]). Previously reviewed studies also showed that the mammalian Rad52 protein can interact with itself, and recently this self-interacting region on MmRad52 has been mapped to the N-terminal region (aa 84–157), using the yeast two-hybrid system [82]. HsRad52 forms complexes both in the presence and absence of DNA [83]. These complexes are heptameric ring structures with a hole in the center [84]. Rad52 ring structures can form on both ssDNA and dsDNA, but it could not be determined whether the DNA in HsRad52/DNA complexes is present in the central hole or on the outside of the ring structure [83]. High concentrations of HsRad51 can displace HsRad52 complexes from ssDNA much more easily than from dsDNA. The authors suggest that one of the functions of the HsRad52/DNA complexes might be to encourage HsRad51 to preferentially form filaments on ssDNA as opposed to dsDNA. Unlike RecA, which has a strong preference for forming filaments on ssDNA, purified HsRad51 forms filaments approximately equally well on both ssDNA and dsDNA, but filament formation on dsDNA inhibits the strand-transfer activity of HsRad51 [67]. The Rad52 protein from yeast also forms ring structures [85], further emphasizing the conservation of the human and yeast Rad52 proteins.

Purified HsRad52 protein was shown to form ring structures at the ends of dsDNA molecules having blunt ends or single-strand overhangs [86]. These complexes are frequently found joining two dsDNA ends of the same or different molecules. It was suggested that the binding of HsRad52 to a DSB may play a role in directing the damage into the HRR pathway (which operates in S and G2 phases), whereas binding of the Ku complex may initiate the NHEJ repair pathway (which is critical in G1 phase) (see discussion in [87]). When HsRad52 is bound to ssDNA, its binding includes the terminal 5′ or 3′ nucleotide [88]. The authors suggest that ssDNA is probably bound in an exposed grove on the surface of HsRad52, and this binding may facilitate annealing of complementary DNA strands by making the bases more accessible for interactions.

3.3. HsRad54 protein

The Rad54 protein is a member of the Snf2 family of proteins, which are related to DNA helicases, and has been shown to increase the rate of Rad51-dependent pairing of ssDNA and dsDNA in vitro (summarized in [1]). Three recent reports using the purified human and yeast proteins have further elucidated the functions of this protein. The purified HsRad54 protein has ATPase activity that is completely dependent on dsDNA but no DNA helicase activity is seen using standard assays [89]. Using a topological assay, the same group found HsRad54 can introduce negative supercoils into a nicked plasmid, and this reaction requires HsRad54's ATPase activity [90]. The authors interpret their data to indicate that HsRad54 is utilizing ATP hydrolysis to unwind dsDNA. They suggest that this activity could promote formation or stabilization of HsRad51-mediated joint molecules. These findings would strongly indicate that HsRad54 is acting downstream of the proteins assisting HsRad51 in forming nucleoprotein filaments. The earlier paper from this group [89] reported that a HsRad54 mutant that can presumably bind ATP, but not hydrolyze it, partially complements the IR and MMC sensitivity of *mmrad54* knockout ES cells, indicating that an ATPase activity may not be essential for HsRad54's function(s).

A study of the yeast Rad54 protein [91] resulted in findings similar to those seen with HsRad54. The isolated yeast Rad54 protein is a monomer in solution but forms dimers and/or oligomers on DNA. ScRad54 assists Rad51 and RPA in homologous DNA pairing, and this activity is completely dependent on the ability of Rad54 to hydrolyze ATP. In a topological assay similar to that described above, Rad54 changes the linking number of circular dsDNA, and this activity is also completely dependent on ATP hydrolysis. They report that their results are "consistent with a model in which a Rad54 dimer/oligomer promotes nascent heteroduplex joint formation via a specific interaction with Rad51 protein and an ability to transiently unwind duplex DNA".

As we previously reviewed [1], the phenotypes of *rad54* mouse ES-cell and chicken DT40 knockout

mutants are not as severe as expected from studies of yeast *Rad54* mutants. The interpretation of these in vivo experiments has been complicated recently by the finding of a second human gene, called *Rad54b*, whose product shows a moderate level of homology (36% identity and longer than Rad54, 747 versus 910 residues) with *Rad54* [92]. Cell lines or mice containing knockouts of *RAD54B*, or of both *RAD54* and *RAD54B*, have not been reported, but a physical association, possibly indirect, between Rad54B protein and Rad51 was demonstrated [93].

3.4. HsRad51 paralogs

As indicated above, vertebrates possess five Rad51 paralogs (XRCC2, XRCC3, Rad51B-D) that share ~20 to 30% amino acid sequence identity with HsRad51 (previously reviewed in [1,11]). In yeast the Rad51 paralogs are Rad55 and Rad57, which form a heterodimer that assists Rad51 in DNA strand exchange, probably by helping it form filaments on ssDNA that is coated with RPA [94]. Previously we reviewed evidence primarily from the yeast two-hybrid system that each human Rad51 paralog interacts with one or more of the others, and two of them interact with HsRad51 (see Fig. 2). Like Rad55 and Rad57, but unlike HsRad51, none of the human paralogs interacts with itself. Many of the interactions first seen in the two-hybrid system have now been confirmed by in vitro experiments using both the baculovirus system and proteins purified from *E. coli* ([95,96] and Albala, personal communication). In vitro experiments have also shown that certain proteins can form two interactions simultaneously [96]. For example, Rad51C can bind to both Rad51B and Rad51D at the same time. These results indicate that these proteins probably form heterotrimers or larger multimers, rather than just dimers. Evidence from HeLa cells suggests that Rad51D (Rad51L3) and XRCC2 form a dimer in vivo, but these proteins are not associated with a higher molecular weight complex, at least in the unirradiated cells examined [95]. These authors also demonstrated that the purified Rad51D protein by itself has ssDNA binding activity, as well as ATPase activity that is stimulated by both ssDNA and dsDNA. The Rad51B protein has likewise been purified and characterized [97]. Unexpectedly, this protein was reported to have protein kinase activity and phosphorylated a number of different proteins, including TP53, cyclin E, and Cdk2. Rad51B was also reported to interact in vitro with TP53, PCNA and Cdc2. Much work remains to be done to determine the exact function(s) of these vertebrate Rad51 paralogs in HRR and/or other pathways.

3.5. Rad50/Mre11/Nbs1 protein complex

In addition to Rad51 and the proteins that associate with it, there is another protein complex in yeast and mammalian cells that probably plays an important role in processing DSBs via HRR. This complex contains the conserved Rad50 and Mre11 proteins, and a third protein (Xrs2 in yeast and Nbs1/p95/nibrin in mammalian cells) that is not well conserved between yeast and mammals [1,4]. In yeast, this complex is clearly involved in HRR [98], but it is still unclear in mammalian cells whether this complex is involved exclusively in HRR, or also in NHEJ. The mutations in this complex seem to resemble mutations in HRR more than mutations in NHEJ. For example, vertebrate mutants of Mre11 and Rad50 are inviable (like Rad51 mutants) and NBS1 mutants have high chromosomal instability [99]. The Rad50 protein has ATPase and DNA binding activities, while Mre11 encodes a 3′–5′ exonuclease, and the Rad50/Mre11 complex has also been shown to have endonuclease activity on hairpin structures (reviewed in [1]). 3′-ssDNA tails have generally been considered necessary for initiating HRR in eukaryotes (Fig. 1). Since Mre11 exonuclease activity produces 5′ overhangs, it is not surprising that some exonuclease-deficient mutations in the yeast *Mre11* gene do not block HRR [98,100]. Many mutations that inactivate the exonuclease activity do block HRR, but these mutations also block interaction with Rad50 and complex formation. In vivo characterization of vertebrate *mre11* and *rad50* mutations has been stymied by the cellular and embryonic lethality of knockout mutations [49,101,102]. Recent in vitro characterization of NBS1 by Paull and Gellert has shown that it assists HsMre11/HsRad50 both in unwinding duplex DNA and in cleaving fully paired hairpin structures [103]. ATP increases both of these activities, and in the Mre11/Rad50/Nbs1 complex only Rad50 binds ATP. Although this complex can unwind duplex DNA, this activity is limited to short stretches of DNA and the

complex does not appear to be functioning as a helicase. Paull and Gellert suggest that that this complex "may function in double-strand break repair as a key organizer that determines the type of processing to be used on double-strand breaks, depending on the structure of the ends at the break and perhaps on the availability of other factors". This model suggests that this complex might assist in recruiting other proteins involved in either HRR or NHEJ.

A central question in DSB repair is what determines which pathway is utilized. As previously discussed [1], it is likely that NHEJ operates efficiently in the G1 phase of the cell cycle and on unreplicated DNA in S phase. One can speculate that once DNA has replicated, the composition of the chromatin might be altered in such a way as to favor the recruitment of proteins that initiate HRR (HsRad51, HsRad52, etc.) rather than the Ku/DNA-PKcs complex. Based on a physical interaction between Ku70 and Mre11, as well as the absence of Mre11 nuclear foci (which are synonymous with Rad50 foci; see below) in *ku86* mutant CHO cells, it was recently proposed that binding of the Ku70/86 heterodimer destines the DSB for repair by NHEJ [104]. Although the relative levels of Ku70/86 and Rad51/52 proteins may partially determine the choice of pathway, other factors, particularly the phase of the cell cycle, are likely to be important.

4. Biological significance of Rad50 and Rad51 foci

4.1. HsRad51 nuclear foci

The mammalian Rad51 protein is found in nuclear foci during S and G2 phases of proliferating cells, and IR exposure increases the number of foci (reviewed in [1]). The biological significance of these foci is still not well understood, but several recent studies have been enlightening. Rad51 foci were shown to include single-strand DNA binding protein and to co-localize to ssDNA following treatment with γ-rays, MMC, or etoposide [105]. Each of these DNA damaging agents is known to induce DSBs, and the processing of DSBs frequently involves the formation of long 3′ single-stranded regions (Fig. 1). The ssDNA in these experiments was localized using an antibody specific to bromodeoxyuridine-containing ssDNA. Rad51 foci have also been shown to accumulate at regions of the DNA in which DNA double-strand and single-strand breaks have been introduced [106]. The results of these studies are consistent with a functional role for Rad51 foci in repairing DSBs. However, another report suggests that Rad51 foci observed cytologically may represent DSBs that are never successfully repaired in the heavily damaged cells [107]. 24 h after irradiation, most of the Rad51 foci are found in micronuclei (chromosomal material encased in nuclear membranes outside of the nucleus) or are associated with long DNA fibers that are eliminated from the nucleus. These authors suggest that visible foci must contain at least 100 fluorescent antibody molecules, and that most sites of successful DNA repair may contain many fewer molecules of Rad51. Therefore, although the Rad51 foci observed cytologically may not be at the sites of successful DSB repair, similar but invisible foci probably form at the sites of completed repair [107].

Recent studies have provided a better understanding both of the proteins that are present within Rad51 foci and the proteins necessary for Rad51-foci formation. In human cells, both Rad54 and Rad54B co-localize with Rad51 after γ-irradiation [93], and similarly mouse Rad54 co-localizes with Rad51 in ES cells treated with IR, MMC, and MMS [90]. Rad54-deficient ES cells fail to form Rad51 foci [90]. However, *rad54* chicken DT40 cells showed *increased* Rad51 foci [43], suggesting an accumulation of Rad51 filaments and possible differences between species or cell types. It is still unclear whether Rad52 colocalizes to Rad51 foci although a mammalian Rad52-GFP partially co-localizes with Rad51 foci following IR (Tan cited in [108]). It was reported that MmRad52 co-localizes with MmRad50 foci [109], but this study did not examine Rad51 foci. On the other hand, unlike *rad54* mutations, mammalian *rad52* mutations do not appear to block Rad51 foci formation (Pastink cited in [108]). As discussed in Section 3.2 (also see Fig. 2), there is evidence that Rad52 may play a role in HRR during the initial processing of a DSB (possibly by the Rad50 complex) before the participation of Rad51. Therefore, it is quite possible that Rad52 may be associated with both Rad50 and Rad51 foci. Similarly, γ-H2AX is associated with both Rad50 and Rad51 foci [110]; γ-H2AX is the phosphorylated form of histone H2AX induced by DNA damage. H2AX is phosphorylated within 1–3 min of DNA damage and appears to

localize at the site of damage prior to either Rad50 or Rad51, leading to the suggestion that γ-H2AX may be involved in recruiting other proteins to this site.

The breast-cancer associated proteins BRCA1 and BRCA2 were previously shown to interact indirectly or directly, respectively, with HsRad51, but have now been shown to interact with each other and to be present in S-phase Rad51 foci in untreated cells [111,112]. Cell lines lacking BRCA2 do not form Rad51 foci [113], whereas tumor cells lacking BRCA1 form Rad51 foci but not Rad50 foci (discussed in Section 4.2). Cells overexpressing BRCA2's BRC repeats, one of the regions involved in binding Rad51, also do not form Rad51 foci and have increased IR sensitivity [63,113]. In apparent contradiction to the human tumor cell results discussed above, Brca1-deficient mouse ES cells do show diminished Rad51 focus formation after exposure to IR or cisplatin [114].

Previously it was shown that Rad51 foci did not form in irs1SF, a CHO *xrcc3* mutant cell line [115], suggesting a role for the Rad51 paralogs in focus formation. Additional evidence for this role comes from experiments showing that IR and MMC-induced foci also do not form in irs1, a V79 *xrcc2* mutant cell line, and foci are restored after transfection with the human *xrcc2* gene (N. Liu, personal comm.). Another V79 mutant in the *xrcc11* complementation group, which has not been sequenced, is also defective in Rad51 focus formation [116]. In addition, focus formation is defective after DNA damage in each of the five chicken DT40 mutants containing a knockout of one of the Rad51-paralogs [43,44]. There have been no reports of whether or not Rad51 paralogs co-localize to Rad51 foci.

Bloom's syndrome (BS) is a genetic disorder associated with greatly increased spontaneous SCE and elevated risk of cancer. The *BLM* gene is defective in BS, and is a DNA helicase of the RecQ helicase subfamily (reviewed in [117]). The BLM protein localizes in nuclear foci in proliferating cells [118], and in mouse meiotic cells BLM co-localizes with Mm-Rad51/MmDmc1 in discrete foci, consistent with a role in meiotic recombination [119]. The PML protein present in promyelocytic leukemia cells has also been shown to form nuclear bodies (also known as ND10 or POD bodies) in mitotic cells (reviewed in [120]), and these bodies co-localize with foci containing the BLM protein [121]. The function of the PML protein is not known, but it does contain a zinc-binding domain known as a RING structure that is necessary for PML body formation. The gene encoding PML is disrupted by chromosomal translocations in acute promyelocytic leukemia patients, and PML is defined as a tumor suppressor (reviewed in [120]). Recently it was shown that the *BLM* transcript and protein are cell cycle regulated, increasing in late S and G2, and are also induced by DNA damage [122]. Both in unirradiated late S and G2 cells and in IR damaged cells, BLM is present in nuclear bodies with PML and Rad51; since most Rad51 foci co-localize with BLM/PML bodies, it appears that many nuclear bodies are identical to Rad51 foci [122]. The relationship of PML bodies to Rad51 foci has been somewhat complicated by the recent finding that NBS1 and Mre11 are also associated with PML bodies in untreated cells [123]. Following DNA damage, NBS1 is only occasionally associated with PML bodies. All of the recent data strongly suggest that many different proteins are associated with Rad51 foci, but a number of these proteins are also associated with Rad50 foci.

4.2. Rad50/Mre11/Nbs1 nuclear foci

Mammalian Rad50/Mre11/Nbs1 nuclear foci form only following DNA damage treatment that induces DSBs (i.e. not UV) and are not seen in the same cells that have Rad51 foci (reviewed in [1,4]). It was suggested that Rad50 foci may either be at the sites of active DNA repair of DSB or at the sites of repair that has been initiated but that cannot be completed [4]. In *nbs1* mutant cells, Mre11/Rad50 foci do not form, and the normal damage-induced phosphorylation of Mre11 does not occur [124]. The kinetics of phosphorylation indicates that it normally precedes focus formation, suggesting that Mre11 phosphorylation may be involved in this process.

There is recent information about some of the other proteins associated with Rad50 foci and about mutations that block their formation. As mentioned above, there is evidence that Rad52 colocalizes with the Rad50 foci [109]. Unlike BRCA2, which associates only with Rad51 foci, BRCA1 associates with both Rad50 and Rad51 foci after DNA damage, and *Brca1* mutant cells fail to form Rad50 foci but exhibit normal Rad51-focus formation [125]. A subsequent

study by a different group using the same *brca1* mutant cell line did not find any effect on Rad50 focus formation [126], so the role of BRCA1 in Rad50 foci is still unclear. BRCA1 forms large foci prior to DNA damage, and only a few of these are associated with HsRad51 [125]. Within 1 h after introducing DNA damage, the BRCA1 foci appear to break down, and subsequently BRCA1 is present in both Rad50 and Rad51 foci [125].

The BRCA1 protein has recently been associated with a very large complex of proteins that has been referred to as BASC (BRCA1-associated genomic surveillance complex) [126]. Based on immunoprecipitation, this complex contains at least 15 proteins: BRCA1, ATM, BLM, the Rad50/Mre11/NBS1 complex, mismatch repair proteins, and the five-protein RFC complex. Several of these proteins were also shown to associate with BRCA1 in foci. The authors suggest that this complex participates in DNA damage recognition, such as damage associated with stalled replication. Particularly since the ATM protein phosphorylates BRCA1 at multiple sites (e.g. Ser^{1423}, Ser^{1524}) in response to IR damage [127], it is notable that *atm* mutant human cells, like *brca1* mutant cells, have markedly reduced numbers of IR-induced Rad50 foci (but increased numbers of Rad51 foci) [128]. The increased Rad51 foci in *atm* mutant DT40 cells agrees with this finding [129].

The hCds1 kinase (Fig. 2) is also present in nuclear foci, and these foci co-localize with BRCA1 foci in unirradiated cells [130]. In response to IR, hCds1 phosphorylates BRCA1 at Ser^{988}, and this phosphorylation is necessary for the separation of these two proteins and dispersal of BRCA1 from its foci. Thus, the IR-induced phosphorylation of BRCA1 by both ATM and hCds1 appears to be necessary for normal DNA repair to take place [127,130]. Much remains to be learned about the nature and significance of nuclear foci in DNA repair.

5. Regulation of HRR

5.1. Kinase cascades controlled by Atm and Atr

Cell cycle "checkpoints" are regulatory processes that ensure orderly progression of events during the cell cycle, e.g. the dependence of initiation of mitosis on the completion of DNA replication [131,132]. When cells experience DNA damage or chemically-imposed inhibition of DNA replication, they respond by activating checkpoint pathways that down-regulate cell cycle progression and coordinate DNA repair processes, as recently reviewed in the context of DSB repair pathways [8]. Damage-specific DNA structures probably activate sensor proteins through post-translational modification and/or conformational change. Activation typically confers increased kinase activity such that DNA-structure information is relayed through signaling pathways involving successive phosphorylations. These kinase cascades lead to inhibition of cyclin-dependent kinases, thereby retarding cell-cycle progression. The molecular basis of the recognition step is still largely unknown, but several participating candidate proteins are identified.

The ATM kinase is one of the key candidate sensors that becomes activated throughout the cell cycle [133], possibly by autophosphorylation, within minutes by IR damage [134–138]. Like DNA-PK, ATM can bind to DNA ends and to irradiated DNA [139,140] and may thereby play a direct role in recognizing damage and signaling to downstream effectors (Fig. 2). In the absence of ATM, checkpoint functions mediated by the inhibitory effects of CDKN1A (p21/CIP1/WAF1) on cyclin-dependent kinases do not operate [138,141,142]. The tumor suppresser protein TP53 is a major player that facilitates the G1 and G2 checkpoints. In response to DNA damage, TP53 levels increase through stabilization, and the protein is activated in its ability to enhance transcription of key genes such as *CDKN1A/p21* and *Gadd45* (reviewed in [143]).

The Nijmegen breakage syndrome, which is phenotypically very similar to AT at the cellular level, is defective in the NBS1 protein, another candidate damage recognition factor [137,138,144,145] and one that is apparently not a kinase. NBS1 is a member of the Mre11/Rad50/NBS1 complex discussed above (see Fig. 2), which was shown to localize specifically to subnuclear regions of DNA selectively damaged by irradiation [145]. Like AT cells, NBS cells have an attenuated TP53 response after IR treatment [146–148] although the induction kinetics of TP53 and CDKN1A differ from those of AT cells [149]. Moreover, in response to IR damage NBS cells differ from AT cells

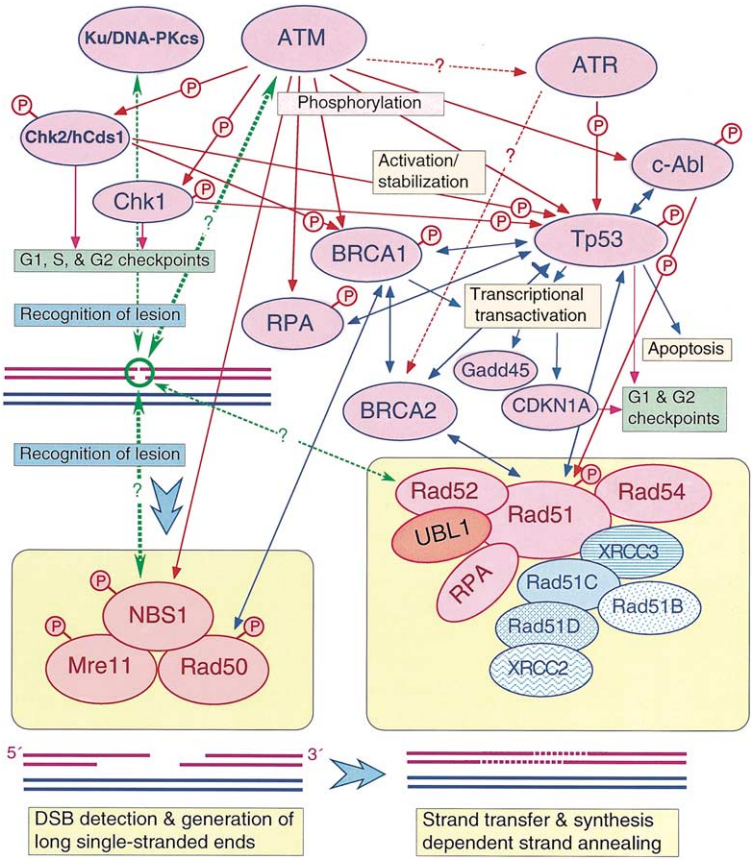

Fig. 2. Regulation of the HRR pathway. In response to IR damage, ATM, ATR, and other sensor proteins not shown (e.g. human homologs of Rad1, Rad9, Hus1, Rad17 [224]) recognize specific alterations in DNA structure, e.g. DSBs. These sensors become activated, possibly through phosphorylation and/or conformational changes, and mediate a hierarchy of reactions that likely recruit ensembles of DNA repair proteins (i.e. Rad50- and Rad51-containing complexes). IR damage initiates phosphorylation of the proteins in the Mre11/Rad50/NBS1 complex by ATM and/or other kinases, and the hyperphosphorylation of Mre11 depends on the presence of NBS1 [124]. The ATR (ataxia telangiectasia and rad3^{+}-related [187]) protein, like ATM and DNA-PK, is a member of the phosphotidyl-inositol-3-kinase family that was identified by sequence similarity to the *Rad3*$^{+}$ gene of *S. pombe*. ATR is implicated in both IR and UV responses based on the finding that overexpression of kinase-defective mutant proteins produces a dominant negative phenotype of radiation sensitivity [188,189]. The BRCA1 and BRCA2 proteins are implicated directly in HRR by their interactions with Rad50 and Rad51, respectively, but also, like TP53, participate in transcriptional activation of critical genes such as *Gadd45* and *CDKN1A* (*p21/CIP1/WAF1*) [178,225,226]. Both BRCA1 and BRCA2 interact with TP53 [227,228] and affect its functions. BRCA1 is reported to stimulate TP53-dependent gene expression [228] whereas BRCA2 inhibits TP53's transcriptional activity [227]. BRCA1 contributes to cell-cycle arrest and growth suppression through the induction of CDKN1A in both TP53-independent and TP53-dependent pathways [229,230]. After IR DNA damage, TP53 functions in sustaining the G2 arrest checkpoint through CDKN1A [231], in addition to its well-documented role in the G1/S checkpoint [232–234]. Chk1 and Chk2/hCds1 kinases negatively regulate Cdc25C (not shown) through phosphorylation [8,173,174,235], which prevents activation of the Cdc2-cyclin B complex and mitotic entry. Chk1 and Chk2/hCds1 also stabilize the TP53 tumor suppresser protein by phosphorylation at Ser^{20} leading to cell cycle arrest in G1 [236–238]. Chk2/hCds1 also appears to act in a DNA replication checkpoint that is independent of ATM [173]. UBL1 is a ubiquitin-like protein that binds to Rad51 and exerts negative regulation, possibly by promoting assembly or disassembly of Rad51-containing complexes [239]. Red arrows indicate phosphorylation, and double-headed blue arrows, as well as overlapping icons, signify direct protein interactions (generally confirmed in vivo). Green arrows indicate candidate proteins that participate in the initial recognition of DSBs and mediate HRR and NHEJ.

by showing more delay in G1/S progression as well as a normal G2/M delay [147,148].

AT cell lines have inherently high spontaneous chromosomal aberrations [150,151] and greatly elevated chromosomal damage after exposure to agents producing DSBs. The lack of a demonstrable defect is DSB repair in early studies may be attributed to very high radiation doses and insufficient assay sensitivity, e.g. [152]. More recently, AT cells have shown partial deficiencies in the extent of rejoining of DNA DSBs measured cytologically in chromatids [153] or DNA molecules using gel electrophoresis methods [154–156]. This partial deficiency in DSB repair may reflect a defect in a specific DSB repair pathway.

Recent genetic studies in chicken DT40 cells suggest that ATM confers radioresistance through the action of HRR and not the NHEJ pathway [129]. This conclusion is supported by the finding that radiation-induced chromosomal aberrations (for cells treated in late S or G2 where repair of DSBs is attributable primarily to HRR [1,16]) were much higher in an *atm ku70* double mutant than in either single mutant or in the *atm rad54* conditionally-double mutant. These differences were also seen in IR survival curves. (The *atm rad54* double mutant was lethal unless constructed as a conditional (tetracycline-dependent) mutant for *rad54*, presumably for the same reason that *rad51* mutants are inviable. A basal level of HRR is apparently required for viability.) Since DT40 cells have unusually efficient HRR relative to NHEJ, it will be important to determine whether this connection between ATM and HRR applies in mammalian cells.

The idea that ATM signaling is required for HRR might appear to conflict with reports of greatly increased capacity (e.g. 100 × elevated) for intrachromosomal recombination in transfected reporter-gene substrates in AT cell lines [157,158]. Differences between species or the nature of the *atm* mutations could account for these apparent discrepancies. In the *atm* mouse model, in which recombination was measured using phenotypic reversion caused by a pop-out event at a 70 kb tandem duplication, the increase was less than 2-fold in the *atm* mutant cells [159]. Moreover, SCE, which reflects homologous recombination [37], is not elevated in AT cells [160,161].

ATM belongs to the phosphotidyl-inositol-3-kinase family like DNA-PK, but ATM does not require DNA ends for in vitro activity [162]. ATM's likely in vivo substrates in response to DNA damage include TP53 [136,163–166], c-Abl [167,168], NBS1 [169–172], Chk1 [8] and Chk2/hCds1 [173–175], RPA [176], BRCA1 [127] (which is known to undergo damage-specific phosphorylation [177]) (see Fig. 2), and CtIP (which participates in regulating *Gadd45* expression in response to IR damage) [178]. The phosphorylation of NBS1 by ATM appears to be critically important in implementing S phase checkpoint functions and HRR [179]. ATM is also required, presumably indirectly, for a TP53 dephosphorylation (Ser^{376}) event that is involved in activation of its sequence-specific DNA binding [180].

The concept of a checkpoint implies that damage-mediated arrest may allow time for cells to repair damage either before DNA replication or mitosis, thereby improving survival and reducing mutagenesis. TP53 has been considered the "guardian of the genome" by mediating the G1 checkpoint [181] and presumably promoting cell survival. However, the TP53 status and duration of arrest at the G1 checkpoint (which is mediated by DNA-damage induced phosphorylation of TP53 by ATM, Chk2/hCds1, etc.) does not broadly correlate with radiation resistance (cell survival). In fact, the absence of TP53 function is associated with *increased* radioresistance in diploid human fibroblasts [182,183] but not in human tumor lines [184]. Moreover, in tumor cells, the length of the G1/S arrest does not depend on the TP53 status [185]. The main function of TP53 may be to maintain genetic stability, through apoptosis or permanent arrest, by preventing cells with damaged chromosomes from continuing to proliferate [183].

As mentioned above, disruption of ATM in chicken DT40 cells, which are null for TP53, causes markedly increased sensitivity to IR [129], as well as loss of G2/M checkpoint activity [186]. Thus, the high IR sensitivity of AT cells may be at least partly caused by the lack of execution of the G2/M checkpoint and its accompanying HRR, whereas the sensitivity of NBS cells may lie more directly in initial damage processing and coordinating HRR.

The recently identified ATR protein [187] also appears to control radiosensitivity and may act in parallel with ATM [188,189] in regulating TP53 but may have non-overlapping functions as well. As with ATM, ATR phosphorylates TP53 [190]. Overexpression of catalytically inactive ATR in γ-irradiated human

fibroblasts selectively inhibited late-phase phosphorylation of Ser^{15} on TP53. High overexpression of ATR results in loss of cellular viability [191], and a knockout mutation in mice results in early embryonic lethality [192]. The arrest of *atr* mutant cells in early mouse embryogenesis, as well as the chromosomal breakage seen in the arresting embryos, resembles several mutants in the HRR pathway (Table 1) and suggests that ATR may also act in this pathway. Notably, in vitro ATR phosphorylated BRCA2 (which interacts with Rad51) whereas ATM did not [162].

Biochemical studies presented evidence for a signaling pathway in which IR-activated ATM facilitates HRR through phosphorylation of c-Abl, which in turn phosphorylates Rad51 at Thr^{315}, thereby promoting Rad51's association with Rad52 to stimulate repair [193]. This IR-stimulated phosphorylation of Rad51 is not seen in ATM- or c-Abl-deficient MEF cultures. However, another study suggested that phosphorylation of Rad51 at Tyr^{54} by c-Abl resulted in inhibition of Rad51's strand transferase activity measured in vitro [78]. Also, genetic studies in DT40 cells do not support the idea that efficient HRR requires a functional interaction between c-Abl and Rad51 [194]. The absence of c-Abl produced by gene knockout did not cause increased radiation sensitivity as measured by chromosomal aberrations or cell survival in either wild-type or ATM-deficient DT40 cells. The deficiency in c-Abl did somewhat suppress IR-induced apoptosis, which is consistent with other studies indicating a pro-apoptotic function for c-Abl [195–197].

As we discussed previously [1], TP53 may play an important role in negatively controlling Rad51's activity. Recent genetic studies based on synthetic direct-repeat or inverted-repeat substrates to measure intrachromosomal recombination, suggest that TP53's putative role as a regulator of HRR is independent of its transactivation functions that regulate the G1/S transition in response to DNA damage [198–200]. It will obviously be important to determine how applicable the findings based on using model recombination substrates are to the efficiency of repair of DNA damage in native chromosomal sequences.

5.2. *Brca1 and Brca2 as participants in HRR*

Recent findings suggest that the BRCA1 and BRCA2 proteins participate in HRR, based on several lines of evidence. The direct functional interaction between ATM and BRCA1 [127] suggests that BRCA1, and BRCA2 by association, may act in a checkpoint pathway as a regulator or mediator of HRR [112]. In addition, the Chk2/hCds1 kinase, which is activated by ATM, also phosphorylates BRCA1 [130] as well as other substrates (Fig. 2). As discussed in Section 2.2, both BRCA1 and BRCA2 contribute to cellular radioresistance. Thus, the pronounced radiation sensitivity of ATM-deficient cells may be at least partially attributable to the failure to properly regulate BRCA1, as well as the G2/M checkpoint kinases Chk1 and Chk2/hCds1 [8]. BRCA2 has well-documented interactions with Rad51 [201–203], and BRCA1 and BRCA2 interact with each other in human cell extracts [111], suggesting that they exist in a complex. Decreased transcription-coupled repair of oxidative damage has also been reported for both human and mouse *brca1* [53,66], but not *brca2*, mutant cells. It is not apparent how this defect might relate to a role for the BRCA1 protein in HRR, raising the possibility of more than one function associated with BRCA1.

6. Involvement of HRR pathway in human cancer

Several recent studies of the genes encoding the HRR enzymatic machinery (see yellow boxes in Fig. 2) point toward the involvement of this pathway in cancer causation. In a study of 127 breast carcinomas [204], LOH detected by polymorphism markers was seen at the regions of Rad51 at 15q15.1 (32% of tumors), Rad52 at 12p13 (16%), and Rad54 at 1p32 (20%). In this study, the BRCA1 (17q21) and BRCA2 (13q12–13) regions had even higher LOH of 49% and 44%, respectively. Moreover, the number of cases in which LOH was seen in two or more regions within the same tumor was always higher than expected by chance, suggesting that these coincident events contributed to the pathological phenotype. LOH in the 12p13 region (*Rad52*) was frequent in another study of breast tumors [205]. These results suggest that there might be mutations associated with the LOH that contributed to the etiology of breast tumors. A search for mutations in Rad54 in breast tumors showing LOH gave negative results [206].

Mutations in *Rad51* were not found in most tumors [207,208]. However, two patients with bilateral

breast cancer had germline Gln150Arg substitution mutations and LOH was present in one tumor [208]. Curiously, *Rad52* germline truncating frameshift mutations (Ser346Ter and Tyr415ter, deletion of 75 and 7 amino acids, respectively) were found in 8% of the human population [209].

In a direct screen of *Rad54* in 132 tumors from breast, colon, and lymph node, three different non-conservative amino acid substitution mutations were identified, one of which was homozygous [210]. In a screen of 45 colorectal cancers and lymphomas for mutations in the *Rad54B* gene at 8q21.3–22, two homozygous non-conservative substitutions were found [92]. No mutations were seen among 80 normal individuals.

Uterine leiomyoma is the most common tumor of smooth muscle cell origin and is often associated with the recurrent balanced translocation t(12; 14) (q13–15; q24). Identification of the breakpoint cluster region has shown that the breakpoints map within the ~900 kb *Rad51B/Rad51L1* gene at 14q23–24 [211,212]. Importantly, this chromosomal region is one of the most commonly deleted regions in gastrointestinal stromal tumors [213], malignant mesothelioma [214], neuroblastomas [215], colorectal carcinoma [216], and nasopharyngeal carcinoma [217].

7. Concluding remarks

The role of HRR in maintaining chromosome stability probably depends heavily on the integrity of the G2/M checkpoint, which helps ensure that DSBs remaining after DNA replication, or produced in G2, are repaired in an error-free manner. When this checkpoint is compromised, there is a much greater likelihood that cells will enter mitosis with chromosome breaks. If the HRR machinery is defective during the period of G2 arrest, the cell must then rely on error-prone NHEJ. This situation will result in chromosomal changes ranging from small deletions and insertions within a chromosome to more deleterious chromosomal exchanges. It is noteworthy that most tumor cells show a reduced ability to cope with IR damage delivered in G2 and measured as chromosomal aberrations in metaphase [218,219]. Specific abrogation of the G2 checkpoint in tumor cells, including those defective in TP53, by using kinase inhibitors is a feasible strategy for cancer therapy, and Chk1 and Chk2/hCds1 are targets for this approach [220–222].

There are still many important unanswered questions in the area of HRR. Several of the most central are: during S and G2 phases, what controls whether a DSB is repaired by NHEJ versus HRR? Which proteins directly involved in HRR are tumor suppressers? Is the human Rad50/Mre11/Nbs1 complex involved only in HRR and not NHEJ? What functional role do the five human Rad51 paralogs play in HRR to minimize chromosome instability? In terms of the regulation of HRR, what are the actual damage-specific DNA structures that are recognized and by what sensor proteins, and what are the initial reactions that activate these sensor proteins?

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